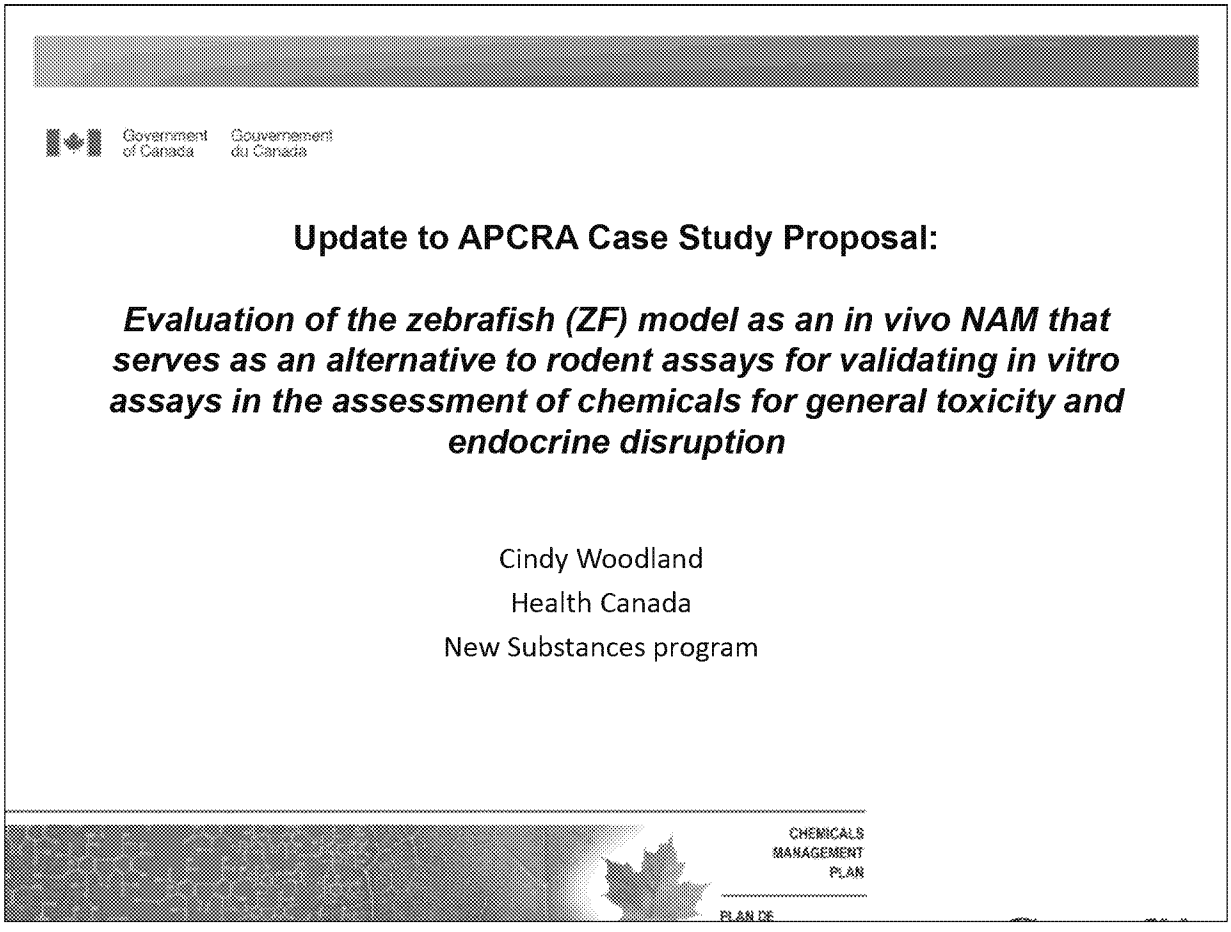
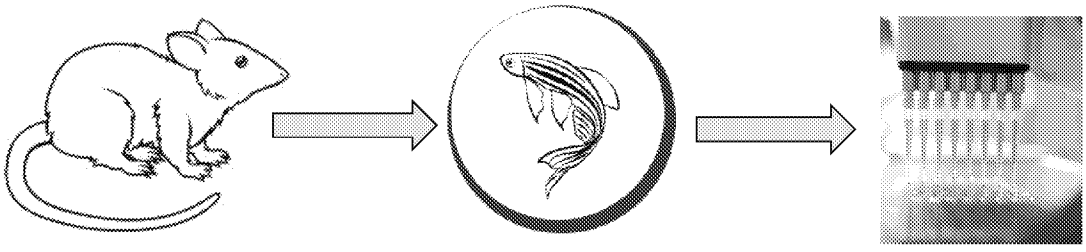


At the APCRA meeting that was held in Ottawa last October, Health Canada proposed this study that explores the utility of the zebrafish model as an alternative to rodent assays, for the purpose of validating the performance of NAMs for predicting endocrine disruption and general toxicity. This project was initiated last summer by Health Canada, in collaboration with the National Research Council of Canada, the NRC, the largest research organization in the government of Canada. The NRC zebrafish testing facility has been in operation since 2007, for which the scientific lead is Dr. Lee Ellis. Dr. Ellis is on the line this morning to answer any questions regarding our zebrafish study.

Purpose of HC-NRC Zebrafish (ZF) Project

The Government of Canada is refining the established ZF model with the goal of optimizing it for eventual adoption as a regulatory tool that will **BRIDGE** the transition from animal to non-animal testing in chemical risk assessment.



Refinements of the existing model include enhancement of evaluations for:

- general toxicity and behavior (neurotoxicity)
- endocrine (adipogenesis) disruption
- gene expression analysis using RNAseq

Compare HC-NRC results with NTP SEAZIT inter-lab validation study results

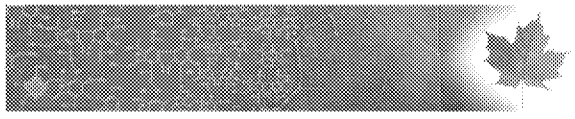
Images taken from:
http://conferences.genetics-gsa.org/zebrafish/index
https://www.labmanager.com/product-focus/2015/05/microplates-for-cell-based-assays

Since the inception of this project, this research has been prioritized for its potential to establish the zf model as a robust regulatory tool in Health Canada chemical risk assessments. is to refine the established zf model Explain what the enhancements are – behavior, (GBT), expansion beyond EATS for ED evaluation (adipogenesis)

ZF study Test Substances

Name	CAS No.	List	Use
Dechlorane Plus	13560-89-9	NTP*	Replacement Flame Retardant
Bisphenol S	80-09-1	NTP*	Replacement Plasticizer
Triphenyl Phosphate (TPhP)	119-61-0	NTP*	Replacement Plasticizer and Flame Retardant
Tricresyl Phosphate (TCrP)	1330-78-5	NTP*	Replacement Flame Retardant
Tris(dichloro-isopropyl) phosphate (TDCPP)	13674-87-8	NTP*	Replacement Flame Retardant
Raloxifene HCL	82640-04-8	ICL, NTP*	Treatment of osteoporosis and breast cancer
Testosterone propionate	57-85-2	ICL, NTP*	Anabolic steroid, treatment of breast cancer
Permethrin	52645-53-1	ICL, NTP*	Human and veterinary insecticide (head lice and scabies)
Thiabendazole	148-79-8	ICL, NDSL, NTP*	veterinary fungicide
Benzophenone	119-61-0	NTP*	UV blocker, flavour ingredient, fragrance enhancer
Bisphenol A	80-05-7	NTP*	Plasticizer
Valproic Acid	99-66-1	NTP*	Anticonvulsant
Aldicarb	116-06-3	NTP*	Pesticide
Amoxicillin	26787-78-0	NTP*	Antibiotic
Pyrene	129-00-0	NTP*	Precursor for dyes, plastics and pesticides
Resorcinol	108-46-3	NTP*	Topical pharmaceutical for treatment of skin disorders
Pyriproxyfen	95737-68-1	NTP*	Veterinary drug for flea control
TBBPA	79-94-7	NTP*	Brominated flame retardant
Propofol	2078-54-8	NTP*	Pharmaceutical sedative
3,4-dichloroanilene	95-76-1	NTP*	Positive control (herbicide precursor)

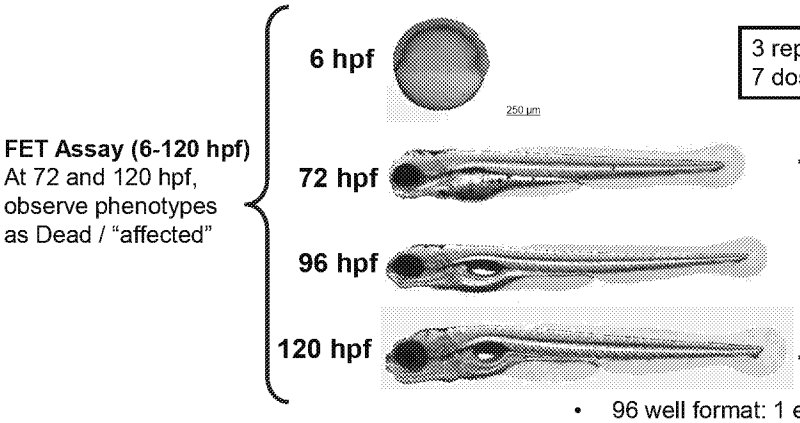
NTP* = List of chemicals selected for inter-laboratory validation study of zebrafish embryo test



NTP substance selection based on:
suggestions from NTP SEAZIT project ,
includes chemicals with a range of physicochemical properties
includes chemicals with a range of developmental effects
many of the chemicals have in vivo (rodent and other zf) reference data

2018-2019 Phenotypic (apical) Evaluation

NRC protocols:
FET - Fish Embryo Toxicity test - Endocrine disruption (adipogenesis)
GBT - General Behavior and Toxicity assay



3 replicates of n=12
7 dose levels + 0.5-1% DMSO control

FET Assay (6-120 hpf)
At 72 and 120 hpf, observe phenotypes as Dead / "affected"

GBT Assay (72-120 hpf)
At 96 and 120 hpf, observe phenotypes as Dead / "affected"
At 120 hpf Behavioral Evaluation
- Distance travelled in light
- Dark "startle" response

- 96 well format: 1 embryo 300 µL per well
- Chorionated - FET
- Buffered media (10 mM pH7.2 HEPES E3 media)
- **Static** exposure
- 28.5C, 14hr:10hr (light:dark)

Images from Sol Gomez de la Torre Canny et al., Dev Dyn. 203:253-310. 2009.

Phenotypic Evaluation for ED and GT

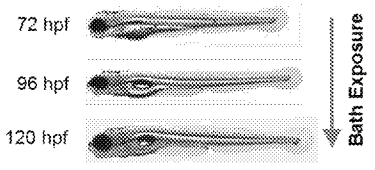
Scoring acronyms

NOE: No observable effect (embryos phenotypically normal for stage, pigment @48hpf, hatched@72hpf)
H: Hatched
UH: Unhatched
LR: loss of lateral recumbancy (after 72hpf)
TSU: Trouble staying upright (after 72hpf)
PCE: Pericardiac edema
HB: Heart beat (eg. No: -, Slow)
BF: Blood flow
LC: Lighter colour
YSE: Yolk surface edema
MA: Melanocyte aggregation
DM: "Donut melanocyte", pigment at outer edges of melanocytes
CM: constant movement
Nec: Necrotic/dead
Unf: embryo appears unfertilized/dead
GH: Grey(cloudy) head
ST: Stubby tail
SH: Small head
BP: Blood pooling
L: L-shaped body
C: C-shaped tail
J: J-shaped tail
Cu: Curled body (as if still in chorions)

/: Missing embryo
X2: two embryos in well

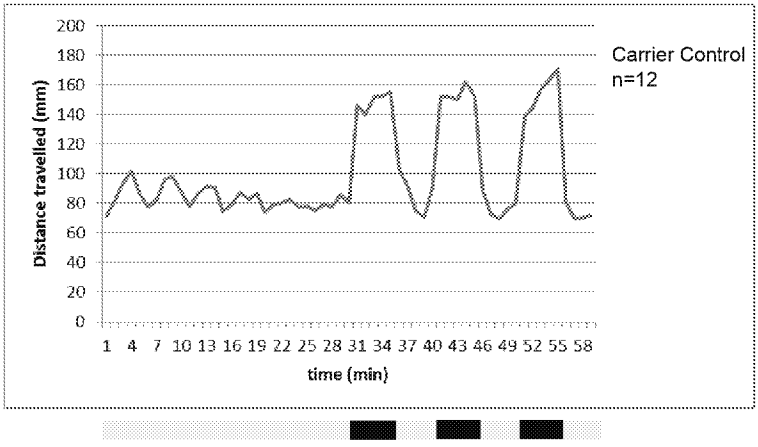
NRC GBT Assay

Quantifiable behavioural measurement



Phenotypes scored: 96 &120 hpf

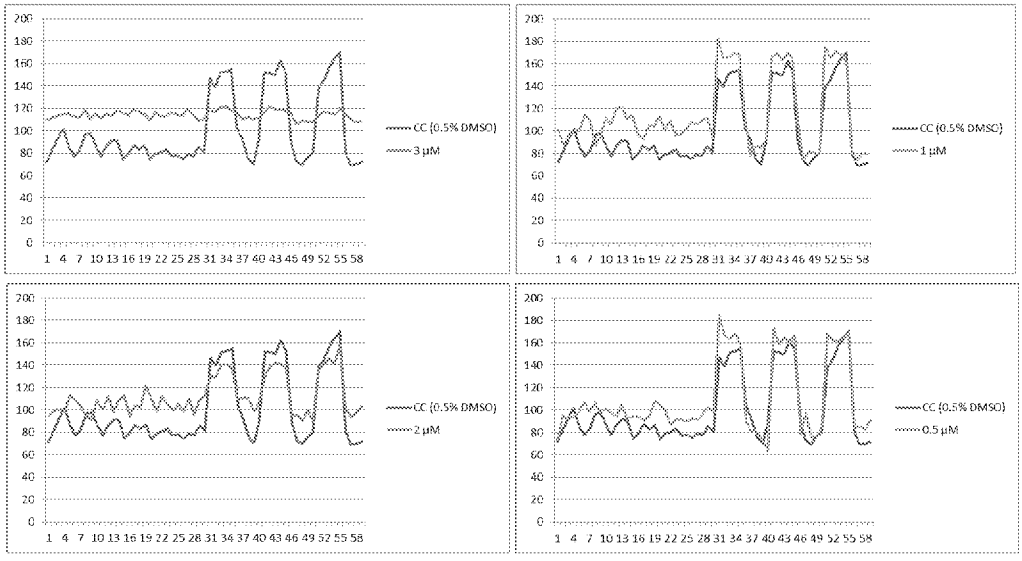
Behavioural Assay: 120 hpf



Light

Dark

GBT Assay: Permethrin



- "Constant movement" phenotype
- Quantifiable behavioural measurement (n=36)

Results - Phenotype

- LC50 – completed for 20 substances
- ED50 – completed for 20 substances
- Behavioral – continuous swimming and startle response evaluation completed for 20 substances, analysis pending

2018-2019 Transcriptomic Evaluation

Dose selection for 3/20 substances (permethrin, BPA, BPS) based on phenotypic EC_{50} of FET. The EC_{20} was calculated and dilutions of 100x and 1000x were made.

Exposure of 6-120 hpf, with 4 replicates
Replicate = 20 larvae – pooled and total RNA extracted for each rep.
Extracted RNA was sent to NRC Saskatoon for RNA sequencing

Substance	EC50	EC20	EC20/100	EC20/1000
Permethrin	3.25 µM	2.31 µM	.0231 µM	.00231 µM

RNA sequencing was performed with analysis pending (collaboration with Jason O'Brien at National Wildlife Research Centre at Carleton University in Ottawa.

The focus will be initially on genes/pathways involved in endocrine disruption, adipogenesis.

2019-2020

Transcriptomics

- Exposure to remaining 7/20 substances, RNA sequencing, and analysis for differential gene expression
- Correlate gene expression results with observed phenotype for ED and GBT

Kinetics – uptake, metabolism and excretion evaluation for 10/20 substances

- FET exposure, 6-120 hpf, sampling at 72 and 120 hpf
- GBT exposure, 72-120 hpf, sampling at 74, 76, 78, 96 and 120 hpf.
- Tissue analysis and bath analysis for parent and metabolites

Compare results with available rodent data for each substance